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Vascular smooth muscle sensitivity to endothelium-derived relaxing factor is different in different arteries

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1 The relaxation responses of pre-constricted pig coronary artery (PCA) and rabbit aorta (RA) without endothelium, to endothelium-derived relaxing factor (EDRF) released from either a PCA or RA with intact endothelium have been studied by use of a bioassay cascade system. Effects of EDRF have been compared with sodium nitroprusside (NaNP) and 8-bromo-cyclic GMP.

2 The time course of changes in cyclic GMP levels in response to EDRF in PCA and RA have also been studied.

3 EDRF (released from a PCA or RA) caused significantly greater relaxation in the PCA than the RA, whether 5-hydroxytryptamine or high extracellular potassium was used as the constrictor agonist.

4 These differences in sensitivity to EDRF were paralleled by NaNP but not 8-bromo-cyclic GMP.

5 Cyclic GMP levels peaked earlier in the RA (30 s) than in the PCA (180 s) but the peak levels were significantly greater in the PCA (2.45 fold) than the RA (1.48 fold).

6 These data show that the previously described differences in EDRF activity between different artery types can be explained in part by differences in the responsiveness of the smooth muscle to EDRF.

Introduction

Endothelium-derived relaxing factor (EDRF) activity varies greatly in different arteries. For example, constrictor responses in the rabbit isolated aorta are largely unaffected by the presence of an intact endothelium, in contrast to rabbit coronary artery preparations, where the presence of endothelium markedly depresses constrictor responses to a variety of agonists (Griffith *et al.*, 1984a). Endothelium-dependent relaxation induced by noradrenaline and substance P has also been found to vary in the large arteries of the dog, with EDRF responses again being greatest in coronary vessels (Angus *et al.*, 1986). Furthermore agonist-induced Ca^{2+} influx is inhibited more by basal EDRF activity in dog isolated coronary preparations than in rabbit aorta (Collins *et al.*, 1986).

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These differences could be due to variation in the amount of EDRF released by different arteries or variation in the response of the smooth muscle to EDRF. EDRF has been shown to exert its effects on vascular smooth muscle by stimulation of the enzyme soluble guanylate cyclase, with a resulting increase in intracellular guanosine 3′:5′-cyclic monophosphate (cyclic GMP) levels (Rapoport & Murad, 1983; Griffith *et al.*, 1985; Busse *et al.*, 1985). Differential responses to EDRF between these two artery types may therefore be due to either a difference in cyclic GMP generation, or a difference in the response to the rise in intracellular cyclic GMP. In the present study we have compared the relaxation responses of the rabbit aorta (RA) and pig coronary artery (PCA) to EDRF using a bioassay cascade system. We used 5-hydroxytryptamine (5-HT), which stimulates receptor-operated calcium channels and

high extracellular potassium (KCl) which stimulates voltage-operated channels, as constrictor agonists. We have also compared relaxation responses to sodium nitroprusside (NaNP) which, like EDRF, acts by stimulation of soluble guanylate cyclase (Katsuki *et al.*, 1977), and 8-bromo-cyclic GMP, a lipid soluble analogue of cyclic GMP which enters the cell. Cyclic GMP levels during relaxation by EDRF have also been measured to investigate further the site of differential EDRF activity in these two arteries.

Methods

Vessel preparation

Rabbit aorta Male New Zealand White rabbits (2–2.5 kg) were killed by a blow to the neck. The descending thoracic aorta was removed and transferred to a beaker containing gassed (95% O_2:5% CO_2) Holman's solution of the following composition (mM), NaCl 120, KCl 5.0, NaH_2PO_4 1.3, $NaHCO_3$ 25, $CaCl_2$ 2.5, D-glucose 11 and sucrose 10. The solution also contained flurbiprofen 10^{-5} M as a cyclo-oxygenase inhibitor to remove the effects of prostanoids.

The aorta was cleaned of adhering connective tissue and sectioned into 2–3 mm wide rings with a fixed blade cutter. Rings were denuded of endothelium by suspending them between two stainless steel hooks and gently rotating a wooden stick against the intimal surface for 10–15 s. For bioassay cascade experiments where an endothelium-intact rabbit aorta donor was used, a 3 cm length of aorta was kept in gassed Holman's solution at room temperature until required. Endothelium-denuded rings were mounted on stainless steel hooks inside an insulating water jacket at 37°C and perfused at 2 ml min^{-1} with gassed Holman's solution at 37°C by use of a Watson-Marlow peristaltic pump (Type MRHE 200). One hook was attached to an isometric force transducer (Ether type UF1 4oz) and tension responses recorded on a Devices chart recorder. Rings were allowed to equilibrate for about 30 min, readjusting to a resting tension of 2 g until stress relaxation no longer occurred. Rings were tested for the successful removal of endothelium by preconstriction with 5-HT (10^{-5} M) and brief (1 min) exposure to the calcium ionophore A23187 (10^{-7} M). Any rings showing a relaxant response to A23187 were considered to have some functional endothelium and discarded. Rabbit aortic rings were then allowed to return to a resting tension of 2 g, adjusting tension if basal tone was altered after the initial exposure to 5-HT.

Pig coronary artery Pigs were killed in a local abbatoir by electrical stunning and exsanguination and the heart removed as soon after death as possible (ca. 10 min). The circumflex coronary artery was immediately dissected free, placed in pre-gassed Holman's solution at room temperature and transported to the laboratory. The coronary arteries were either used within 45 min of collection or stored overnight in gassed Holman's solution at 2°C for use the following day. The coronary artery was cleaned of adhering connective tissue, cut into rings and denuded of endothelium as previously described for rabbit aorta. Similarly a 3 cm length of endothelium intact coronary artery was kept in gassed Holman's solution until required. Rings were mounted on stainless steel hooks as for rabbit aorta but allowed to equilibrate for 60 min at a resting tension of 5 g. All rings were tested for the absence of endothelium as described for rabbit aorta, and allowed to return to a resting tension of 5 g, adjustment being made for changes in basal tone as necessary.

Bioassay system

Large side branches of a 2–3 cm length of rabbit aorta or pig coronary artery were tied off with silk thread. This endothelium-intact donor vessel was placed in a 5 ml chamber containing gassed Holman's solution at 37°C, and cannulated at each end. Endothelium-denuded pig coronary artery and rabbit aortic rings were perfused with Holman's solution at 2 ml min^{-1} via a stainless steel tube mounted in a similar 5 ml chamber filled with Holman's solution at 37°C. When required, the donor vessel could be perfused with Holman's solution at 2 ml min^{-1} and the perfusate allowed to drip onto the recipient rings by moving the donor chamber outlet into position above them (Figure 1).

Experimental protocol

Responses to EDRF The rings of pig coronary artery and rabbit aorta were preconstricted with 5-HT (3×10^{-6} M) or high KCl (26 mM and 34 mM respectively) infused post-donor by use of an LKB (type 2132) peristaltic pump. The concentration of agonists used was that which produced 95% of maximum constriction in this system, i.e. the EC_{95} value for the agonist (data not shown). The composition of the Holman's solution was not altered before the addition of KCl. Since the EC_{95} values for 5-HT in both PCA and RA were similar, the ring preparations could be mounted in parallel thus ensuring exposure to identical concentrations of EDRF released from the donor vessel. After constrictor responses had stabilised the donor vessel was perfused with Holman's solution containing the calcium

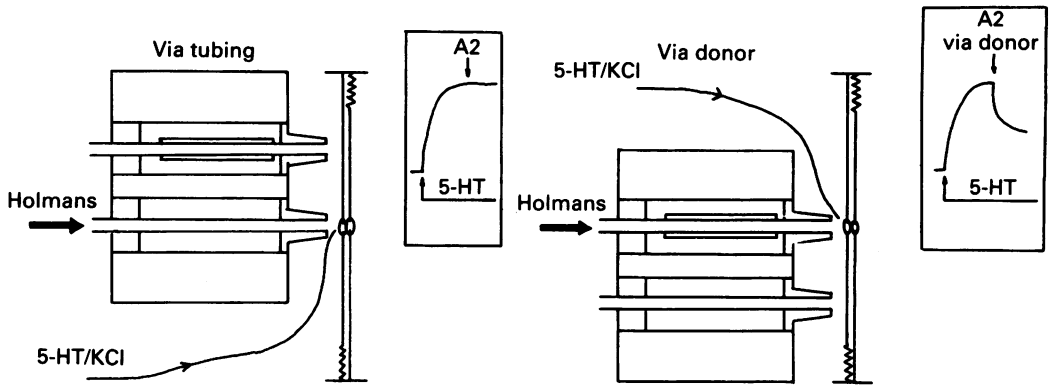

Figure 1 Diagrammatic representation of bioassay-cascade apparatus. Left-hand side shows the endothelium-denuded recipient vessels perfused with Holman's solution via a stainless steel tube. The recipient vessels were constricted with 5-hydroxytryptamine (5-HT) or KCl infused after the tubing. The left-hand inset shows that the constrictor-responses of the recipient vessels were unaffected by the calcium ionophore A23187 (A2). The right-hand side shows the recipient vessels perfused with Holman's solution via the endothelium-intact donor vessel. The right-hand inset shows EDRF-induced relaxation of the pre-constricted recipient vessels by A2 infused through the donor vessel.

ionophore A23187 3×10^{-7} M to release EDRF; this concentration of A23187 was found to induce maximal EDRF release from both PCA and RA and did not alter constrictor responses to 5-HT. This EDRF-containing perfusate was then allowed to drip onto the recipient rings. The amount of EDRF perfusing the recipient rings could be altered by varying the transit time between the donor and recipient vessels from 5 to 30 s. Relaxation responses to EDRF were expressed as a percentage of the initial constriction to 5-HT or KCl. Overnight storage of the PCA preparations rendered them slightly, but significantly less sensitive to 3×10^{-6} M 5-HT. Tension developed to 5-HT in the unstored and stored rings being 8.8 ± 0.3 g ($n = 44$) and 7.5 ± 0.3 g ($n = 42$) respectively ($P < 0.05$). Tension developed to KCl, however, was not significantly altered by overnight storage ($n \geqslant 7$).

EDRF release and maximum relaxation responses to EDRF were similar in the unstored and stored preparations ($n \geqslant 11$). Each experimental group contained similar numbers of unstored and stored ring preparations.

Responses to NaNP and 8-bromo-cyclic GMP Concentration-response relationships of endothelium-denuded coronary and aortic rings were constructed by use of the bioassay-cascade apparatus. The rings were perfused via the control (stainless steel) tube and NaNP or 8-bromo-cyclic GMP added to the Holman's solution in a cumulative manner. The recipient rings were pre-constricted with 5-HT as for the EDRF studies. Relaxant responses were expressed as a percentage of the initial constriction.

Measurement of cyclic GMP Endothelium-denuded rings of pig coronary artery and rabbit aorta were mounted for EDRF bioassay as described earlier. After exposure to EDRF for intervals between 10 s and 300 s, the tension on the rings was released, and they were transferred to liquid nitrogen. This removal procedure took less than 3 s. Control rings were collected for cyclic GMP assay after preconstriction with 5-HT and exposure to A23187 alone.

Rings were homogenized in ice cold 6% trichloroacetic acid (TCA) and after centrifugation the cyclic GMP in the supernatant was measured after removal of the TCA with 0.5 M tri-*n*-octylamine (dissolved in 1,1,2 trichlorotrifluorethane). The residue after centrifugation of the homogenate was estimated for protein content (after dissolving in 1 M NaOH for 1 h) by the method of Bradford (1976). The cyclic GMP content of each ring was measured with a commercially available radioimmunoassay kit (New England Nuclear) and expressed as pmol mg^{-1} protein.

Statistical analysis

Concentration-response curve slopes and EC_{50} values for EDRF, NaNP and 8-bromo-cyclic GMP were calculated from a line fitted to the straight part of the concentration-response curve by a least squares minimisation procedure. Results were

analysed by Student's t test for unpaired data and considered significantly different when $P < 0.05$. Data are presented as mean ± s.e.mean.

Drugs used

5-Hydroxytryptamine (creatinine sulphate complex), calcium ionophore A23187 (calcium, magnesium mixed salts), sodium nitroprusside and 8-bromo-guanosine 3':5'-cyclic monophosphate were purchased from Sigma Chemical Co. Ltd., Poole, Dorset. Flurbiprofen was a gift from the Boots Company PLC, Nottingham.

Results

Responses to endothelium-derived relaxing factor

Figure 2a shows relaxant responses of PCA and RA to EDRF when pre-constricted with either 5-HT or KCl (EC_{95}). 5-HT increased tension in the PCA and RA rings to 8.5 ± 0.9 g and 3.4 ± 0.3 g which relaxed by 89 ± 4% and 35 ± 5%, respectively, when perfused with EDRF released from a PCA donor by A23187 (Figure 2a). In those experiments where KCl was used to preconstrict the recipient vessels, the PCA and RA rings could not be mounted in parallel since the EC_{95} to KCl was different for each artery, i.e. 26 mM for PCA and 34 mM for RA, which increased tension by 10.7 ± 1.2 g and 3.4 ± 0.1 g respectively. EDRF released from a PCA by A23187 relaxed the PCA and RA rings by 37 ± 9% and 8 ± 4% respectively.

Responses of the PCA and RA to varying concentrations of EDRF are shown in Figure 2b. The $t_{1/2}$ of EDRF was calculated as described previously (Griffith *et al.*, 1984b) and in the present study was 10 s. The EDRF concentration after the shortest transit time was considered as unity and all other concentrations expressed proportionally. At each concentration of EDRF achieved, relaxation in the PCA rings was approximately twice as great as in the RA rings.

Relaxation to EDRF released from RA by A23187 was also studied in PCA and RA pre-constricted by 5-HT (EC_{95}). In these experiments, 5-HT increased tension in the PCA and RA by 6.5 ± 0.8 g and 3.1 ± 0.2 g respectively and relaxed by 95 ± 16% and 29 ± 8% ($n = 5$) with EDRF released from the RA.

Responses to sodium nitroprusside

Figure 3 shows concentration-response curves to relaxation induced by NaNP in PCA and RA rings pre-constricted with 5-HT (EC_{95}). 5-HT increased

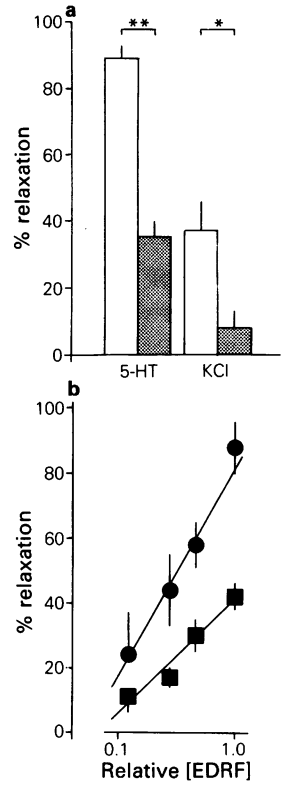

Figure 2 (a) Mean % relaxation of pig coronary artery (PCA) (open column) and rabbit aorta (RA) (shaded column) pre-constricted with 5-hydroxytryptamine (5-HT) ($n \geq 6$) and KCl ($n \geq 6$) to EDRF released from an endothelium-intact PCA by A23187; vertical lines indicate s.e.mean. Relaxation in the PCA was significantly greater than in the RA. * $P < 0.01$; ** $P < 0.001$. (b) Mean % relaxation of PCA (●) and RA (■), pre-constricted with 5-HT, to varying concentrations of EDRF released from an endothelium-intact PCA by A23187. Each point is the mean of at least 4 experiments with s.e.mean indicated by vertical lines. The slope of the concentration-responses was significantly steeper in the PCA ($P < 0.01$).

tension in the PCA and RA rings by 8.5 ± 0.8 g and 2.6 ± 0.2 g respectively. The curve for the PCA had a significantly steeper slope than that for the RA (51.9 ± 2.7 and 36.2 ± 1.7 respectively, $P < 0.001$), and a significantly lower EC_{50} ($5.6 \pm 1.5 \times 10^{-8}$ M and $1.83 \pm 0.52 \times 10^{-7}$ M respectively) ($P < 0.05$). However, the asymptotes for the two preparations were not significantly different.

Responses to 8-bromo-cyclic GMP

Figure 4 shows concentration-response curves to relaxation induced by 8-bromo-cyclic GMP in PCA

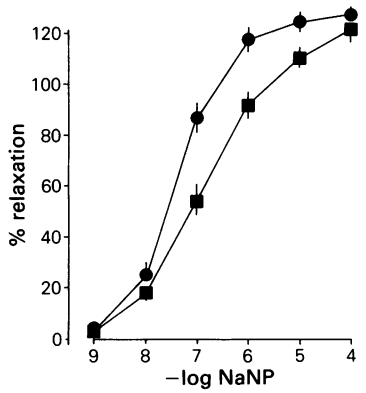

Figure 3 Concentration-responses of mean % relaxation induced by sodium nitroprusside (NaNP) in pig coronary artery (●) and rabbit aorta (■) pre-constricted with 5-hydroxytryptamine ($n \geqslant 11$); s.e.mean indicated by vertical lines.

and RA rings pre-constricted with 5-HT (EC_{95}). 5-HT increased tension in the PCA and RA rings by 8.4 ± 1.0 g and 3.6 ± 0.7 g respectively. The curve for the PCA had a significantly steeper slope than that for the RA (74.4 ± 6.3 and 50.8 ± 3.7 respectively) ($P < 0.01$) and a significantly higher asymptote ($125 \pm 6\%$ and $103 \pm 6\%$ respectively) ($P < 0.05$). There was no significant difference in the EC_{50} values between the two arteries.

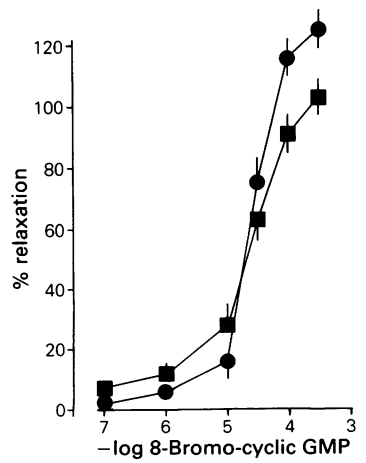

Figure 4 Concentration-responses of mean % relaxation induced by 8-bromo-cyclic GMP in pig coronary artery (●) and rabbit aorta (■) pre-constricted with 5-hydroxytryptamine ($n = 5$); s.e.mean indicated by vertical lines.

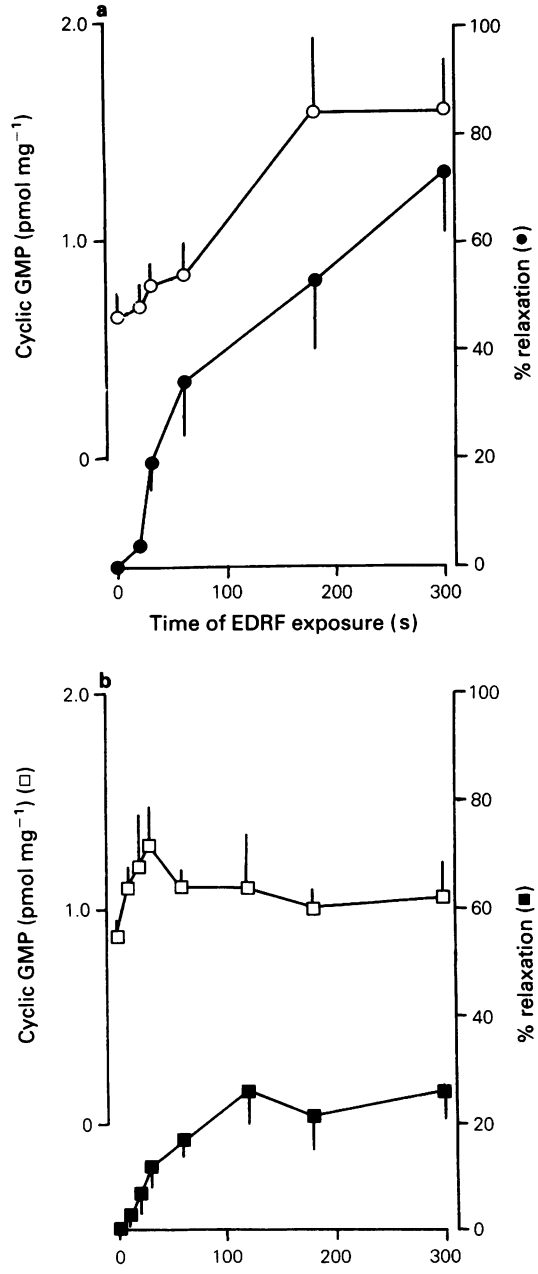

Figure 5 Graphs showing cyclic GMP levels (open symbols) and % relaxation (closed symbols) in pig coronary artery (a) and rabbit aorta (b) pre-constricted with 5-hydroxytryptamine, after exposure for various time intervals to EDRF released from a pig coronary artery by A23187. Each point represents the mean of at least 6 experiments; s.e.mean indicated by vertical lines.

Levels of cyclic GMP

Figure 5 shows EDRF-induced changes in cyclic GMP levels in PCA and RA rings pre-constricted with 5-HT (EC_{95}). 5-HT increased tension in the PCA and RA rings by 8.3 ± 0.3 g and 3.1 ± 0.1 g respectively. A23187 (3×10^{-7} M) was used to release EDRF from a PCA in the bioassay cascade system. Over the 300 s time period studied, peak cyclic GMP increased by 2.45 fold in the PCA rings which was significantly greater than the 1.48 fold increase in the RA rings ($P < 0.05$). Peak levels were achieved after 180 s in the PCA and after 30 s in the RA. The relationship between % relaxation and log cyclic GMP levels was linear in the PCA rings throughout the 300 s studied ($r = 0.82$) but in the RA rings correlation between relaxation and log cyclic GMP was found over the first 30 s only ($r = 0.95$) (Figure 5).

Discussion

This study shows that differences in EDRF activity between the RA and PCA can be explained in part by differences in their responsiveness to EDRF. This difference was paralleled by a similar difference in their responsiveness to sodium nitroprusside which, like EDRF, acts by stimulating soluble guanylate cyclase. The EDRF concentration-response curves for PCA and RA extend over one log unit only, being limited by the amount of EDRF released from a PCA donor in this bioassay system. They are comparable to the nitroprusside concentration-response curves over the range 10^{-8} to 10^{-7} M, extending between ca. 20% and 90% for PCA and ca. 15% and 45% for RA for both EDRF and nitroprusside.

The differences in responsiveness to EDRF between the two arteries was present whether they were contracted with 5-HT or KCl. It is unlikely therefore that the reason for the differences can be related to the constrictor agonist used. It is worth noting that EDRF-induced relaxation in the KCl-constricted vessels was approximately half that in the 5-HT-constricted vessels. These findings are similar to those previously described and support the observation that vascular smooth muscle preparations preconstricted by activation of voltage-operated calcium channels do not respond as well to agents which increase levels of cyclic GMP as preparations pre-constricted by activation of receptor-operated calcium channels (Collins *et al.*, 1988).

The response to EDRF remained greater in the PCA regardless of whether a PCA or RA was used as the donor vessel, thus showing that EDRF is not species specific. The data also indicate that since the responses of both recipient vessels were similar whether a PCA or RA was used as donor, the relative amounts of EDRF released from both vessels were similar when maximally stimulated by the calcium ionophore A23187. However, unpublished observations (Christie & Lewis) suggest that flow and receptor-agonist-stimulated release are different in these two vessels.

Comparison of the concentration-response curves of 8-bromo-cyclic GMP shows no difference in the EC_{50} concentrations between the two vessels. Although the slope of the concentration-response curve for the PCA was significantly steeper than that for the RA, it is doubtful whether such a small difference has any biological significance. These findings with 8-bromo-cyclic GMP are in marked contrast to those obtained with EDRF and nitroprusside and are unlikely to explain the differences in responsiveness between these two vessels.

Comparison of the cyclic GMP data shows marked differences in the time-course of the levels achieved between PCA and RA. In PCA the rise in cyclic GMP was gradual with a maintained plateau whereas in RA an early peak occurred followed by a lower maintained level. It is also worth noting that for any given level of intracellular cyclic GMP generated by EDRF, the relaxation achieved in the PCA is greater than that achieved in the RA. This contrasts markedly with relaxation induced by 8-bromo-cyclic GMP which was similar in the two arteries.

The findings of this study imply firstly that the cyclic GMP generating systems of the PCA and RA are different and secondly that the response of the contractile proteins to intracellularly generated cyclic GMP is greater in the PCA than the RA (Pfitzer *et al.*, 1984). The fact that little difference can be shown between the two vessels to exogenously applied 8-bromo-cyclic GMP, would suggest that this agent affects different intracellular pools of cyclic GMP from stimulants of soluble guanylate cyclase like EDRF and nitroprusside.

In conclusion, the findings of the present study explain, at least in part, the previously described differences in EDRF activity in different arteries.

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